Rapid Procedure for Determination of Nicarbazin Residues in Chicken Tissues

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A procedure is described for the quantitation of nicarbazin residues in chicken tissues. The method includes extraction of tissue with chloroform-ethyl acetate-dimethyl sulfoxide (50 + 50 + 0.8), adsorption on neutral alumina, and subsequent elution of the residues with methanol-pH 6.0 phosphate buffer (1 + 1). Extracts are separated on a 15 cm, 5 μm C_{18} column with methanol-pH 6.0 phosphate buffer (6.5 + 3.5) as the mobile phase. The dinitrocarbanilide portion of the complex is detected and quantitated with an electrochemical detector in the reductive mode. Recoveries, based on dinitrocarbanilide, were greater than 95% in liver, breast, and thigh muscle tissues fortified with 0.25-8.0 ppm nicarbazin.

Nicarbazin, an equimolar complex of 4,4-dinitrocarbanilide (DNC) and 2-hydroxy-4,6-dimethyl-2-pyrimidinol (HDP), is used for the prevention of coccidiosis in chickens. Chickens excrete DNC more slowly than HDP (1), hence most analyses for residues in tissue have focused on this portion of the complex. Indeed, the official Food and Drug Administration method for nicarbazin residues involves pulse polarographic determination of the isolated DNC (2). Liquid chromatography in conjunction with UV detection has also been used, as part of multiresidue procedures (3, 4), for detecting DNC residues in tissue extracts. These latter methods, like the polarographic procedures, are capable of detecting DNC residues below the established tolerance level of 4 ppm (5). However, the procedures are rather cumbersome and lengthy and therefore are not suitable for routine screening of chicken tissues. Because of these limitations of the available methods, a simple, rapid procedure has been developed for determining nicarbazin residues in edible chicken tissues above or below the tolerance level. The method, based on a modification of a previously published procedure for the quantitation of Zoalene and its metabolites in chicken tissues (6), uses solid phase extraction and liquid chromatography-electrochemical detection (LC-EC) of DNC in tissue extracts.

METHOD

Reagents and Apparatus

(a) *Solvents.*—Ethyl acetate and methanol (distilled in glass, American Burdick and Jackson, Muskegon, MI 49442); chloroform, Baker Analyzed reagent (J. T. Baker Chemical Co., Phillipsburg, NJ 08665); dimethyl sulfoxide (DMSO) (Fisher Scientific Co., Fairlawn, NJ 07410); *N,N*-dimethylformamide (DMF) (Aldrich Chemical Co., Inc., Milwaukee, WI 53201).

(b) *Nicarbazin.*—Merck and Co., Inc., Rahway, NJ 07065. Dinitrocarbanilide (DNC) was obtained by partitioning into ethyl acetate from an aqueous solution of nicarbazin according to the procedure of Michielli and Downing (2).

(c) *Tissue homogenizer.*—Polytron (Brinkmann Instruments Inc., Westbury, NY 11590).

(d) *Centrifuge.*—Refrigerated, IEC CENTRA-7R. Rotor No. 822A (International Equipment Co., Needham Heights, MA 02194).

(e) *Neutral alumina.*—Brockman Activity I, 80–200 mesh (Fisher Scientific Co.). Insert 6 mm glass bead into 5 mL pipet tip (Rainin Instrument Co., Woburn, MA 01801). Layer glass bead with 0.5 cm sand (Washed and Ignited for Boats, J. T. Baker Chemical Co.) and 1.25–1.5 cm bed of neutral alumina, firmly packed by gently tapping top of pipet tip. Add 0.25 cm sand. Wash column with two 2 mL portions of $CHCl_3$–ethyl acetate (1 + 1) before use.

(f) *Liquid chromatograph.*—Altex Model 100A pump (Altex Scientific Inc., Berkeley, CA 94710) connected to BAS Model LC-45 amperometric detector (Bioanalytical Systems Inc., West Lafayette, IN 47905): glassy carbon electrode −0.8 V vs Ag/AgCl, 50–200 nA full scale. Altex Model 210A

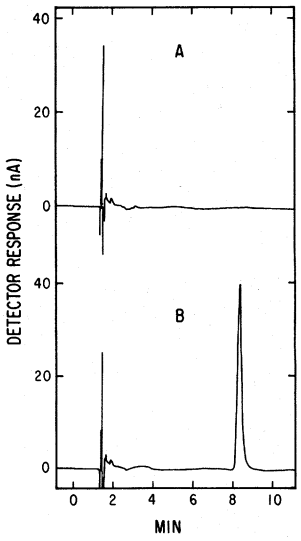

Figure 1. LC chromatograms of (A) extract of control liver and (B) extract of liver fortified with 4.0 ppm nicarbazin. Electrochemical detection: potential −0.8 V; attenuation 100 nA full scale.

Table 1. Percent recovery of DNC from chicken tissues fortified with nicarbazin[a]

Tissue	Nicarbazin added, ppm	Recovery,[b] %
Liver[c]	0.25	97.5 ± 2.3
	0.50	96.8 ± 4.2
	1.00	99.1 ± 2.8
	2.00	93.0 ± 3.3
	4.00	96.0 ± 2.2
	8.00	96.5 ± 2.7
Breast[d]	0.50	97.5 ± 2.8
	1.00	100.5 ± 2.1
	2.00	100.6 ± 1.0
	4.00	98.4 ± 3.6
	8.00	98.4 ± 4.6
Thigh[d]	0.50	94.2 ± 5.7
	1.00	104.9 ± 1.0
	2.00	100.4 ± 3.0
	4.00	101.6 ± 2.1
	8.00	100.6 ± 1.6

[a] Based on analysis of extract equivalent to 0.25 g tissue.
[b] Mean ± standard deviation.
[c] Five different tissues at each concentration.
[d] Three different tissues at each concentration.

sampling value with 50 μL loop. Column: 15 cm × 4.6 mm id 5 μm Supelcosil LC-18 (Supelco, Inc., Bellefonte, PA 16823). Mobile phase: methanol–pH 6.0 phosphate buffer [0.05M KH_2PO_4 adjusted to pH 6.0 with NaOH; 0.001 mole EDTA (6.5 + 3.5)] purged with helium. Elute samples isocratically at 1.0 mL/min flow rate. Sampling technique: Purge 2 mL sample in 9 mL vial for 2 min with helium to exclude oxygen from system. Draw ca 0.8 mL sample through capillary tubing into loop, by way of vent, with hypodermic syringe inserted in sampling valve in "load" position.

Preparation of 4.0 ppm Drug Standard

Prepare stock solution A of nicarbazin in DMF to contain 2.0 μg/μL. Dilute 5 μL solution A to 10 mL with methanol–pH 6.0 buffer (6.5 + 3.5) in volumetric flask (solution B). Let solution B stand in dark, with occasional shaking, for 1 h. Dilute solution B (1 + 1) with methanol–pH 6.0 buffer prior to injection onto LC column. For other concentrations, prepare solution A accordingly and dilute as described.

Recovery Studies

Spike drug-free frozen, ground tissues by injection with 5 μL of appropriate DMF solutions of nicarbazin. Hold spiked samples at −10°C for 18 h prior to analysis. Use unspiked tissues as control.

Determination

Place 2.5 g frozen, ground tissue into 50 mL polypropylene centrifuge tube. Let sample partially thaw. Add 20 mL $CHCl_3$–ethyl acetate–DMSO (50 + 50 + 0.8) and blend 45 s with tissue homogenizer at moderate speed. Centrifuge 5 min at 3500 rpm. Remove and discard aqueous layer. Recover solvent, filter it through small plug of glass wool packed tightly in 4 mL disposable Pasteur pipet, and collect filtrate. Pass 2 mL filtrate through neutral alumina column. Wash sides of column and alumina with 3.25 mL $CHCl_3$ in 0.75, 0.75, 0.75, and 1.0 mL increments. Remove excess $CHCl_3$ from column with air pressure and maintain pressure until column dries as evidenced by disappearance of moisture on outside of column. Continue air pressure for additional 3 min. Elute column with pH 6.0 phosphate buffer–methanol (1 + 1); collect first 2 mL effluent in 2 mL volumetric flask. Shake stoppered flask thoroughly and use disposable Pasteur pipet to transfer effluent to 9 mL screw-cap specimen vial. Inject 50 μL sample onto LC column according to procedure described above.

Results and Discussion

Comparative LC studies with nicarbazin and DNC standards revealed that nicarbazin dissociated completely in solution B within 1 h, and gave a uniform DNC chromatographic peak; the HDP portion of the complex is not detected by the LC-EC system in the reductive mode. Detector response to DNC was linear at the concentrations used in this study (r = 0.9997), and the solutions were stable for at least 5 days when stored at room temperature in the dark. At higher methanol–pH 6.0 buffer ratios (4:1), dissociation of the nicarbazin complex occurred more slowly and appeared to recomplex after standing several days. At lower methanol–buffer ratios (1:1), high concentrations of DNC precipitated out of solution on storage. Potential solubility problems with the drug controls can be avoided by closely following the procedures described here or by using DNC as the control and calculating the concentration of nicarbazin accordingly.

Figure 1 depicts a typical chromatogram of an extract of chicken liver spiked with 4 ppm nicarbazin (tolerance level) and its corresponding unfortified control tissue. Because of detector specificity, samples can be injected successively on the LC system since no late eluting peaks have been observed. In addition, LC studies on a variety of electrochemically active nitro-containing drugs and their metabolites showed no interference with DNC on the chromatograms. Tissue control samples run at low attenuations reveal a background peak, originating from the alumina and/or solvents, which elutes at the retention time of DNC. Under the conditions of this study, the background, equivalent to 0.02–0.04 ppm nicarbazin, limits the lowest detectable level to 0.1–0.2 ppm (5× background). For quantitative results at concentrations above 8.0 ppm, it is recommended that less than 2 mL tissue extract be used in the assay to eliminate solubility problems which may occur in the drug controls and/or alumina effluents at higher concentrations. However, the lesser amount of extract should be diluted to 2 mL with $CHCl_3$–ethyl acetate (1 + 1) prior to analysis.

Table 1 summarizes the recovery studies on livers (N = 5), breasts (N = 3), and thigh muscles (N = 3) spiked with nicarbazin concentrations ranging from 0.25 to 8.0 ppm. The overall mean recoveries and standard deviations were 96.5 ± 3.3% for liver, 99.1 ± 2.9% for breast, and 100.4 ± 4.5% for thigh muscle. The near quantitative recoveries of DNC, which is known to complex with tissue components, can be attributed to (*1*) the relatively high ratio (8:1) of extracting solvent to tissue; (*2*) the use of a definite amount (2 mL) of the recoverable extract (15–17 mL) in the analytical procedure; and (*3*) the presence of DMSO in the extracting solvent. In the latter case, Wood and Downing (7) reported that DMSO complexes with DNC to "swamp out tissue complexation" and allow for complete extraction of the residue from incurred tissues. Indeed, we observed a 15% decrease in recoveries when DMSO was omitted from the extracting solvent, which suggests that complexation also occurs in fortified tissues.

Using the procedure described here, an analyst can concurrently prepare 2 tissue extracts for LC analysis every 45 min; LC analyses can be performed at 10 min intervals.

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